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(54) Title: A METHOD FOR THE EVALUATION OF ANTIVIRAL DRUGS

(57) Abstract

This invention discloses the utility of gelatin sponge as an *in vivo* matrix for numerous viral models for the evaluation of antiviral drugs. Human cytomegalovirus (HCMV) or any other human virus that is difficult to culture in a non–human host, such as HCV, HIV and HPV, may all be used with this system for evaluating antiviral drugs.

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A METHOD FOR THE EVALUATION OF ANTIVIRAL DRUGS

Field of the Invention

This invention relates to the field of viral infections and disease and novel methods for evaluating drugs that may suppress or control those viral infections or diseases.

Information Disclosure Statement

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References listed above are incorporated by reference into this document.

Summary of the Invention

This invention discloses the utility of gelatin sponge as an *in vivo* matrix for numerous viral models for the evaluation of antiviral drugs. Cytomegalovirus or any other human virus, preferably those difficult to culture in a non-human host, may be used. Specific examples provided herein include: a xenograft murine human cytomegalovirus (HCMV) model, a human immunodeficiency virus (HIV-1) model, a hepatitis c virus (HCV) model and a human papilloma virus (HPV) model for the evaluation of potential antiviral compounds. The methods described here can provide *in vivo* efficacy data for potential drug candidates. Cytomegalovirus or any other human viruses that are difficult to culture in a non-human host may be used.

Target cells, used in the gelatin sponge, can be any cell of human or animal origin but they will usually be human. Human cell examples are human cell lines; primary cells obtained from human blood (such as lymphocytes and monocytes) as well as from human tissues (such as hepatocytes from liver biopsies, macrophages from lung washing or peritoneal fluid). Immortalized cell lines are most conveniently used but sometimes other cells may grow better. Here, we discovered that human foreskin fibroblast (HFF) cells are better for the HCMV assay because these cells grow better than human embryonic lung (HEL) cells. The HFF cells were initially prepared from fresh human tissue, but they can be cryopreserved and sub-cultured for many passages. The gelatin sponge can be implanted subcutaneously or at sites other than subcutaneous, such as within the peritoneal cavity.

Any type of antiviral compound could be discovered using the gelatin sponge technique described herein. Such antiviral agents can include organic or inorganic compounds with specific antiviral effects. An example of such an antiviral compound is ganciclovir. In addition to "typical" organic or inorganic compounds, products of

natural or recombinant technology such as polyclonal and monoclonal antibodies, cytokines and interferon may be evaluated and discovered using the procedures disclosed here.

This invention describes a process for selecting or evaluating compounds having antiviral activity comprising: exposing target or test compounds to virus growing in cells which are inoculated into gelatin sponge, which has been implanted into mammals, and then evaluating the effect of the target compounds on the growth of the virus. The cells implanted into the gelatin sponge may be, and usually will be selected from human cells. These human cells are selected from: primary cells obtained from human blood or cells from human tissues. The human blood cells can be selected from lymphocytes and monocytes. When cells from human tissue are selected they may be hepatocytes from liver biopsies or deceased organ donors, macrophages from lung washing or peritoneal fluid.

The virus may be selected from any infectious virus, especially those that infects humans, such as human cytomegalovirus, human immunodeficiency virus (HIV), hepatitis c virus (HCV), and human papilloma virus (HPV). Any mammal may be suitable as gelatin sponge implant species including rodents, such as mice or rats, including Sprague-Dawley rats, as well as other species including dogs and cats.

Genetically immune-deficient mammals may also be used, such as genetically immune-deficient mice, or rats, including nude mice, SCID or severe combined immunodeficiency mice, and mice or rats administered with a immuno-suppressive drug regimens, such as Sprague-Dawley rats administered with cyclophosphamide.

Immuno-suppressive drugs include, but are not limited to cyclophosphamide, methotrexate, azathioprine, cyclosporine and anti-lymphocyte globulin. The cells that are infected with the virus are typically human cells. Cells from human tissues may be derived from human embryonic lung tissue, where the virus is human cytomegalovirus. Cells may be from lymphocyte cell lines, where the virus is HIV or HCV. Cells may be from hepatocytes harvested from liver tissue, where the virus is HCV. Typically, but not always, the gelatin sponge is implanted into the mice or rats separately. Sometimes the gelatin sponge is implanted before the virus is inoculated into the cells in the sponge. Sometimes the gelatin sponge is implanted after the virus is inoculated into the cells in the sponge. Sometimes the inoculated cells are inoculated into the previously implanted gelatin sponge. Sometimes the inoculated

cells are inoculated into the gelatin sponge before it is implanted. But the timing of the steps and decisions as to which steps are made in what order can all be altered to best achieve the desired result of creating a good viral assay. One skilled in the art would be expected to make variations from the specific steps described here.

In a preferred embodiment the described process takes the following steps, the process of using gelatin sponge for the evaluation of antiviral agents, comprising the following:

a) implanting gelatin sponge into a mammal,

b) infecting human cells with a virus,

c) injecting viral infected human cells into the gelatin sponge,

d) exposing the mammal to target compounds

e) evaluating the effect of target compounds on the viral infected cells in the gelatin sponge.

The order may be changed, for example, the gelatin sponge may be inplanted after it is injected with the viral infected human cells.

The evaluation may be the selecting of specific compounds having antiviral activity from the target compounds. In a more preferred embodiment the virus is selected from human cytomegalovirus (HCMV), HIV or HCV, the mammal is a rat or a mouse and the gelatin sponge is GELFOAM® and it is implanted into the mouse or rat subcutaneously. The rat may be a Sprague-Dawley rat and it may be immunosuppresed with cyclophosphamide.

Background of the Invention

The usual progression in the development of an anti-infective agent is to establish the efficacy and therapeutic index of a drug in cell culture then proceed to animal protection studies, pharmacokinetic and toxicity profiles. For many viruses, the human pathogen can cause a disease in animals comparable to the human infection. However, some diseases, like HCMV are highly host specific and they do not infect other animal species. Surrogate animal models using mice, guinea-pig, and rats infected with the respective endogenous CMV have been employed to evaluate antiviral drugs and study the pathogenesis of CMV infections. Unfortunately, animal CMVs may differ significantly from HCMV in drug susceptibility, nucleotide homology, and replication time. For example, murine CMV is susceptible to acyclovir but HCMV is relatively resistant. These differences between HCMV and other

animal CMVs have led to various animal model studies using xenograft and matrix implantation to study pathogenesis and evaluate potential HCMV drugs.

Mocarski, *et al.*, above, reported a xenograft-based animal model of HCMV using severe combined immunodeficient (SCID) mice implanted with human fetal tissue. In this model, human fetal thymus and liver tissue were introduced under the kidney capsule as a conjoint implant. One to six months after implantation, the fetal tissues under the kidney capsule were inoculated with virus. Mocarski showed that implanted fetal tissue will support viral growth and treatment with ganciclovir can reduce viral replication. Another xenograft technique employed small fragments of human fetal retinas that were transplanted into the anterior eye chambers of SCID mice, DiLoreto, *et al.*, above. The grafts were inoculated with virus one week after transplantation.

Although these models allow HCMV replication, they are often costly, time consuming, and technically complex. They also require human fetal tissue which is not readily available to most laboratories and may be difficult to retrieve from the host animal. In addition, because of the relative lack of nucleotide homology between human and animal CMVs, immunodeficiency virus, hepatitis virus or other viruses, surrogate models may not be useful for the evaluation of inhibitors that are highly specific to human CMV, human HCVs, human immunodeficiency virus or other viruses

Alternatives to xenograft models are techniques that involve implantation of macroencapsulated human cells into rodents. Two such models of viral infection have recently been reported that employ non-tissue matrices to entrap cells in mice. (Allen LB, , *et al.*, above and Hollingshead, , *et al.*, above). The agarose plug method used human embryonic lung cells infected with HCMV in vitro and suspended in liquified agarose. Implants were prepared by drawing small volumes of the cell-agarose mixture in plastic syringes and allowing the agarose to solidify. Agarose plugs were implanted into mice either via the ip or subcutaneous (sc) routes. Unfortunately, agarose implants tend to elicit excessive inflammatory responses that lead to breakdown of the agar matrix that complicate retrieval of the agarose plugs (Allen LB, *et al.*, above).

To improve on the agarose approach, Hollingshead, *et al.*, above, developed a method using PVDF hollow fibers filled with HIV-1 infected human lymphoid cells.

The fibers were heat sealed and implanted into SCID mice ip and sc. Hollingshead showed that HIV-1 will replicate in cells trapped in hollow fibers and that antiviral drugs can be evaluated in this model.

Detailed Description of the Invention

Gelatin sponge, such as GELFOAM® sterile sponge, is a licensed medical device intended for application to bleeding surfaces as a hemostatic. For animal studies, small pieces of gelatin sponge have been used to serve as an easily retrievable receptacle for injected tumor cells, see Akporiaye, above. Here, we present the utility of gelatin sponge as an *in vivo* matrix for numerous viral models for the evaluation of antiviral drugs. Cytomegalovirus or any other human virus that is difficult to culture in a non-human host may be used. Specific examples provided herein include a xenograft murine human cytomegalovirus (HCMV) model and a human immunodeficiency virus (HIV-1) model. A Hepatitis C Virus (HCV) model and a human papilloma virus (HPV) model for the evaluation of potential antiviral compounds is also described. The methods described here can provide *in vivo* efficacy data for potential drug candidates.

The most elaborately described example model herein is the HCMV model. Because of the increasing numbers of individuals with compromised immune functions, infection with human cytomegalovirus (HCMV) is now recognized as a leading viral opportunistic infection. For example, about 20% of AIDS patients develop HCMV retinitis that may progress to blindness if untreated. In bone marrow transplant, interstitial pneumonia due to HCMV infection is responsible for greater than 15% mortality. In addition, HCMV infection is known to cause graft rejections that account for about 25% mortality in renal transplant patients.

Two nucleoside inhibitors of HCMV (ganciclovir, foscarnet) have been widely used in the treatment of HCMV disease. Prolonged use of ganciclovir is often associated with serious side effects such as anemia, neutropenia and irreversible testicular damage. Treatment with foscarnet may result in nephrotoxicity and hypocalcaemia. Moreover, prolonged use of both these drugs has also led to the emergence of drug-resistant HCMV strains. Therefore, it is important to develop novel chemotherapeutic agents against HCMV, especially those with a different mechanism of action.

Due to the strict species specificity of this virus, animal models of HCMV infections have not been available to study pathogenesis or to assess antiviral drugs. Various antiviral agents against HCMV have been evaluated in surrogate models in which mice, rats, and guinea-pigs were infected with the respective endogenous CMV. However, because of the relative lack of nucleotide homology between human and animal CMVs, surrogate models may not be useful for the evaluation of inhibitors that are highly specific to HCMV. The problems described here have led us to develop this unique model for evaluating potential antiviral drugs using HCMV and human target cells.

Using the procedures and information provided above one should be able practice all aspects of this invention. The following information is provided to further characterize, illustrate and exemplify the invention.

In one embodiment of the invention, HCMV infected cells (human embryonic lung, HEL, cells or human foreskin fibroblast, HFF, cells) were injected into gelatin sponges (gelatin sponge) that had been surgically implanted subcutaneously in mice. At various days after drug or placebo treatments, gelatin sponge implants were harvested and the homogenized samples were tittered by a viral plaque assay. Our results showed that HCMV can survive and replicate in human cells within an *in vivo* gelatin sponge environment. Also, mice treated with ganciclovir either intravenously (iv) or intraperitoneally (ip), showed significantly lower viral titers than the placebo treated group. A rat model showed similar results. These models provide an inexpensive method for the in vivo evaluation of lead drug candidates against HCMV, examples with other viral systems are also provided.

The models described herein can be used for immune-competent mice and rats capable of normal host defence, and, in addition to this, similar models can be set up with any genetically immune-deficient mice or rats such as nude mice, SCID or severe combined immunodeficiency mice. Also suitable are normal mice or rats administered with various immuno-suppressive drug regimens such as cyclophosphamide, methotrexate, azathioprine, cyclosporine and anti-lymphocyte globulins.

Definitions and Materials

Antiviral Agent is any compound, agent, chemical or any defined and characterized agent that controls or suppresses the viability, growth or infectivity of a virus.

Cytomegalovirus. Can be any strain. Here, the Davis strain of Cytomegalovirus (VR-807) was acquired from American Type Culture Collection (Rockville, MD). The original source of the virus was isolated from a liver biopsy of a three-month old girl with microencephaly. Viral stocks were propagated in human embryonic lung cells (HEL 299) and culture supernatants were aliquoted in 2.5 mL amounts and frozen at -800C.

Ganciclovir (GCV) or (Cytovene), [9-(1,3-dihydroxy-2-propoxymethyal) guanine] was purchased from a hospital pharmacy. The lyophilized drug was reconstituted with sterile saline and stored at 40C. Fresh stock was prepared every other day. Ganciclovir was administered ip or iv once a day at 50 mg/kg body weight. The control mice were inoculated with equivalent volume of saline. Treatments were started 2 h after inoculation of infected cell and continued daily for six consecutive days. Therapies were discontinued the evening preceding sample collection. Four to six mice constituted each treatment group.

Gelatin sponge is any liquid-permeable, water-insoluble, gelatin sponge having the general physical characteristics of a sponge but being absorbable by animal bodies. A gelatin sponge can be made according to US Patent 2,465,357, incorporated by reference. The gelatin sponge used in the examples herein was obtained from Pharmacia & Upjohn, Co., Kalamazoo, MI., USA, and is given the trademark name GELFOAM®. Gelatin sponge may be inoculated with cells. The cells may be infected with a virus. The cells may be introduced into the gelatin sponge either before or after the gelatin sponge is introduced into a body cavity of a mammal. The cells may be infected with a virus either before or after the cells are introduced into the gelatin sponge.

HEL 299 cells (CCL 137), derived from human embryonic lung tissue, were obtained from American Type Culture Collection. Cell cultures were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% heated-activated fetal bovine serum (FBS), 100 units/mL of penicillin G, 100 Tg/mL of

streptomycin and 2 mM L-glutamine. Cell monolayers were passaged according to conventional procedures using 0.25% trypsin plus 1 mM EDTA-4Na.

Immortalized cell lines are most conveniently used but sometimes other cells may grow better. Here, we discovered that human foreskin fibroblast (HFF) cells are better for the HCMV assay because these cells grow better than human embryonic lung (HEL) cells. The HFF cells were prepared from fresh human tissue, rather than from an established cell line.

HIV may be any strain of infectious Human Immunodeficiency Virus.

Inoculation or inoculate means the introduction of one thing into another. Inoculation or inoculate is not limited to the medical definition of the introduction of a microorganism or infective agent into the tissues of living animals and plants, although that is included in the definition of the word used herein. Here it also includes the introduction of any one material (living or not) into another, such as the introduction of cells into gelatin sponge or the introduction of gelatin sponge into a mammal. It includes the introduction or infection of virus into cells, but it can also include the introduction of a glass marble into a gelatin sponge.

Rodent is any member of the rodent family including rats, mice, gineapigs, rabbits, marmots, etc.

Twelve-week-old female ICR mice were procured from Charles River Laboratories and acclimated for one week before the start of the experiment. The out-bred mice were housed in filter top polycarbonate cages with food and water provided *ad libitum*. All animal studies were conducted in AAALAC approved animal research facilities in compliance with USPHS Policy for the Humane Care and Use of Laboratory Animals. Biosafety Level 2 or 3 precautions were observed.

Additional embodiments of the invention are provided to further characterize and exemplify the invention, but the embodiments described herein should be considered illustrative and are not intended to limit the invention.

HCMV Mouse Model.

Procedures. Mice were anesthetized with sodium pentobarbital at a dose of 40 mg/kg body weight or a mixture of ketamine, xylazine and acepromazine at 1.8 mg/kg body weight. The animal's dorsal side was shaved and cleansed with povidone iodine solution-70% ethanol. A 15x15x10 mm gelatin sponge (gelatin sponge) was pre-moistened in sterile saline then implanted subcutaneously through an incision in

the dorsoscapular region of the recipient mouse. Surgical glue or stoppers were used to seal the wound opening. All surgical procedures were performed under aseptic conditions. Two days after gelatin sponge implantation, 2×10^6 HCMV infected cells in 0.06 mL culture medium were injected into the sponge matrix through the skin. On selected days after cell injection, mice were euthanized with CO_2 gas and the sponges were retrieved through a skin incision. Extracted gelatin sponge sponges were individually homogenized with a glass tissue grinder and the collected homogenate samples were frozen at -80^0C. The specimens were thawed and gelatin sponge debris was removed by centrifugation. Virus titers were determined on the supernatant by plaque assay on monolayers of HEL 299 cells with a limit of detection of 30 plaque-forming units (pfu) per mL.

It now appears that human foreskin fibroblast (HFF) cells are better for the HCMV assay because these cells grow better than human embryonic lung (HEL) cells. The HFF cells were prepared from fresh human tissue.

Virus Replication. A virus replication study was designed to investigate the survival and growth of HCMV and HEL 299 cells within a gelatin sponge environment. In vitro, cells were exposed to virus 20 h before gelatin sponge inoculation. Sponge matrices were then collected from two mice at 6 h and days 1, 3, 5, 7, 9 after cell injection. Samples were analyzed for virus by plaque assay. Other experiments were performed to further explore the mechanism of this model. In these studies, implants inoculated with infected and uninfected cells were removed from mice 1 and 4 days after cell inoculation. The gelatin sponges were cut in half and slide imprints were prepared. Separately, some implants were snap-frozen in liquid nitrogen for preparation of frozen sections. The slides were air dried and fixed in acetone. A direct fluorescent antibody test was done on the imprints and cross-sections using a mouse anti-HCMV monoclonal antibody conjugated to fluorescein isothiocyanate. Specimens were also submitted to histology for hematoxylin-eosin staining.

HCMV Rat Model

Male Sprague-Dawley rats (125-150 grams) were obtained from Charles River Laboratories. Animals were housed in wire cages at 5-6 rats per cage. Water and food were provided *ad libitum* throughout the day. All animal studies were

conducted in AAALAC approved animal research facilities in compliance with USPHS Policy for the humane care and use of laboratory animals.

Viruses and Cell Culture. HFF cells were purchased from Clonetics (San Diego, CA). Cells were maintained in Corning T-150 cm^2 vented tissue culture flasks in Dulbecco's MEM (DMEM) supplemented with 10% FBS. HCMV (Davis strain) was obtained from ATCC and virus stocks were prepared. For virus infection, subconfluent monolayers of HFF cells were incubated with stock virus diluted in DMEM with 5% FBS and incubated at an m.o.i. of 0.01 at 37°C for 20 hours prior to inoculation of implanted Gelfoam (unless otherwise indicated). Following incubation, the virus inoculum was removed and cells were washed briefly with Dulbecco's PBS without Ca^{++} and Mg^{++}. Cultures were treated with 0.25% trypsin/EDTA and suspended cells were washed with DMEM/5% FBS. Cells were pelleted by centrifugation at 250 g in a Beckman GS-6R swinging bucket centrifuge and resuspended in DMEM/5% FBS. Cells were counted in an improved Neubauer hemocytometer, pelleted and resuspended in DMEM/5% FBS to 2.33 x 10^7 cells/mL.

Gelfoam Implantation and Inoculation. A 12 mm diameter by 1 cm piece of Gelfoam was soaked overnight in DPBS at 4°C to pre-wet the Gelfoam. Rats were anesthetized with xylazine (67.5 mg/kg), ketamine (3.4 mg/kg), and acepromazine (0.675 mg/kg). The dorsal area of the rat was shaved and cleansed with 70% ethanol and a small incision was placed in the dorsoscapular region. The Gelfoam was inserted within the subcutaneous region approximately 2 inches distal to the incision. The incision was closed with Nexaband S/C (VPL). Two days following implantation, rats were anesthetized as previously described and the Gelfoam was inoculated with 60 µl of HCMV-infected HFF cells at 2.33 x 10^7 cells/mL. Upon termination of the study, rats were euthanized with CO_2 followed by cervical dislocation. The implanted Gelfoam was aseptically removed, homogenized in 2 mL DMEM/5% FBS and stored at -80°C until virus titration was performed.

Virus Titration. Corning 24-well tissue culture plates were seeded with 5 x 10^4 HFF cell/well the day prior to virus titration. Gelfoam homogenates were thawed, vortexed briefly, and centrifuged at 1000 g in a Beckman GS-6R centrifuge with a swinging-bucket rotor. Supernatant was diluted 1/3 or 1/30 in DMEM/5% FBS

and 0.1 mL was used to inoculate a well of a 24-well tissue culture plate. All samples were performed in duplicate. Wells were overlayed with 0.7% carboxymethylcellulose in DMEM/7.5% FBS 2 hours after inoculation. Plates were incubated at 37°C/5% CO_2 for 6-7 days. Monolayers were stained with 0.1% crystal violet/10% paraformaldehyde and plaques enumerated.

Compounds. Ganciclovir (Cytovene-IV, Roche Laboratories) was obtained from Bergen-Brunswick as a vial of sterile powder. Compound was dissolved and diluted in Dulbecco's PBS on the first day of dosing. Single day aliquots were made and stored at -20°C until day of use. Cidofovir (Vistide, Gilead Laboratories) was obtained as a sterile solution at 75 mg/mL. Compound was diluted in Dulbecco's PBS and stored at room temperature throughout the dosing period. Cyclophosphamide (Sigma C-7397) was obtained as a dry powder in an ISOPAC container and rehydrated at 20 mg/mL with sterile saline.

Results. Initial studies using the rat as a host for the HCMV Gelfoam model demonstrated highly variable levels of HCMV replication (data not shown). Harvested Gelfoam appeared granulamatous which was not observed when using the mouse as a host. This granulamatous appearance was suggestive of a host inflammatory response. To test this hypothesis, an immunosuppressant, cyclophosphamide, was evaluated at various dose levels for its effect on HCMV titers in the rat Gelfoam model. An increase in viral yield was observed with increasing doses of cyclophosphamide. The highest viral titers were observed in rats dosed with cyclophosphamide at 100 mg/kg. The mean $\log_{10}$ HCMV titer was 1.5 logs higher at 100 mg/kg when compared with saline-treated controls. However, signs of toxicity were observed in rats dosed with cyclophosphamide at 100 mg/kg which was manifested as a decreased rate in weight gain and hair loss beginning at day 6 (data not shown). Viral titer was one log higher among rats treated with cyclophosphamide at 50 mg/kg when compared with saline control groups. Toxicity was not apparent within this group of rats. Due to this lack of apparent toxicity, further Gelfoam® experiments were performed using cyclophosphamide pre-treatment at 50 mg/kg.

A time-course was performed to study the kinetics of HCMV replication in cyclophosphamide-treated rats. Rats were administered a single bolus dose of cyclophosphamide at 50 mg/kg immediately following implantation. HCMV was

inoculated in the Gelfoam 2 days post-implantation. Although little or no virus was detectable within 24 hours following inoculation, peak viral titers were achieved with both groups 3 days after inoculation. In immune-competent animals, mean viral titers decreased rapidly from peak levels of 65,300 pfu/mL (day 3) to 7,150 pfu/mL (day 5). In immune-suppressed animals, greater peak viral titers (89,725 pfu/mL) were observed on day 3 after cell inoculation which decreased slightly to 61,750 pfu/mL by day 5 after inoculation. The cyclophosphamide model also showed reduced variability in viral titers.

The antiviral efficacy of ganciclovir (GCV) was compared between rats pre-treated with cyclophosphamide (50 mg/kg) and rats pre-treated with saline. GCV was administered subcutaneously at 50 mg/kg/day b.i.d. for 5 days. As demonstrated in the previous experiment, HCMV titers were higher on day 5 among animals pre-treated with cyclophosphamide than rats pre-treated with saline (mean pfu=10020 and 37620 for saline and cyclophosphamide pre-treated rats, respectively). HCMV titers were the same for both groups treated with GCV (mean titer = 300). A greater net reduction in virus titer following GCV therapy was observed in the cyclophosphamide treated group (2.2 log reduction) versus the non-cyclophosphamide saline group (1.6 log reduction). Based on the increased sensitivity and the reduced variability within the cyclophosphamide treated groups, we incorporated cyclophosphamide pre-treatment at 50 mg/kg to evaluate the efficacy of HCMV antivirals in the rat Gelfoam model.

A dose titration was performed with GCV to determine in vivo efficacy in the rat Gelfoam® model. Rats were treated with cyclophosphamide at 50 mg/kg as described above. GCV was administered at 100, 50, 25, and 0 mg/kg/day, intraperitoneally, b.i.d. following inoculation of the Gelfoam® with HCMV-infected HNDF cells. Gelfoam® was harvested on day 5 after virus inoculation. GCV at 100 mg/kg/day significantly reduced ($p<0.05$) HCMV titers in the Gelfoam (98% reduction) and markedly reduced HCMV titers at 50 and 25 mg/kg/day with an 84% and 58% reduction, respectively.

Cidofovir (CDV) is a recently approved HCMV antiviral with potent antiviral activity. This compound also demonstrates intracellular stability which reduces the frequency of dosing. *In vitro*, CDV has been demonstrated to be 20-fold more potent against the Davis strain of HMCV than GCV (Kern ER, Value of animal models to

evaluate agents with potential activity against human cytomegalovirus. *Transplantation Proceedings* 1991: 23(3 Suppl 3):152-5). The *in vivo* efficacy of CDV against HCMV was evaluated using the rat Gelfoam® model. A single bolus dose of CDV was administered intraperitoneally at 50, 10, 2 or 0 mg/kg immediately following inoculation of the implanted Gelfoam® with HCMV-infected HNDF cells. Again, Gelfoam® was harvested 5 days post-inoculation. A single dose at 50 mg/kg significantly reduced viral titers by 96% ($p<0.05$) and reduced virus titers by 85% at 10 mg/kg. A single dose of CDV at 10 mg/kg resulted in the same degree of virus reduction as multiple doses of GCV at 50 mg/kg/day. These data demonstrate improved potency of CDV when compared with DHPG and correlate with the improved in vitro activity associated with CDV.

The improved *in vivo* potency of CDV when compared with GCV has also been demonstrated using other animal CMV models of *in vivo* replication. Kern ER, above, 1991, demonstrated that CDV significantly reduced mortality rates in the murine MCMV model at 0.6 mg/kg when dosed b.i.d. beginning six hours post infection; whereas the same dose of GCV did not significantly reduce mortality. A significant reduction in mortality did not occur until GCV was administered at 5.6 mg/kg b.i.d. Using a rat CMV model, Stals FS, De Clercq E, Bruggeman CA, *Antimicrobial Agents Chemother*. 1991; 35(11):2262-6, reported that a single dose of CDV at 5 mg/kg administered 6 hour post-infection led to a significant reduction in virus titer. This reduction was comparable to that achieved with DHPG at 20 mg/kg/day for 5 days. Kloover JS, Vanagt WY, Stals-FS, Bruggeman CA, *Antiviral Res*. 1997; 35(2):105-12, reported that treatment with one dose of CDV at 20 mg/kg effectively reduced virus titers in an experimentally induced intracranial rat CMV (RCMV) infection in rats that were immunosuppressed by total body X-irradiation.

The efficacious dose of both GCV and CDV were higher in our rat Gelfoam® model when compared to the reported efficacy of these drugs in the murine and rat CMV models. However, for the purpose of drug evaluation, it should be pointed out that the current model is responsive enough to readily produce a dose response for GCV and to rank order active antivirals. Therefore, the model presented here should provide an important advantage in selecting a candidate drug with equal or greater potency than GCV.

Some of the more obvious factors that may affect drug activity in the Gelfoam® model are the strains of virus and animal used, the time of initiation of drug treatment and drug metabolism. Another factor that may influence the activity of a compound is the time of initiation of antiviral therapy.

Finally, the decreased antiviral efficacy observed in the Gelfoam® model may be due to a less efficient localization of parenterally administered drug to target cells within the Gelfoam® environment. Vascularization of target organs likely play a significant role in drug delivery to the site of infection. The target tissues or organs in animal models using an endogenous virus to infect its natural host would naturally be more vascularized than in models in which synthetic implants such as Gelfoam® are used. However, even with a native virus-host system, different target sites may display different levels of viral inhibition due to the disposition of the compound in the animal.

Acute and Cell-to-Cell Virus Infection Models

For the evaluation of antiviral drugs, two gelatin sponge murine models were developed that employed different mode of virus infection. In the acute method, cells were incubated in vitro with cell-free virus for twenty hours. They were washed to remove unabsorbed virions then injected into the gelatin sponge matrix. In the cell-to-cell approach, infected cells after six days of infection were mixed with uninfected cells at a 1:4 ratio before being injected into gelatin sponge. Mice were treated ip or iv with ganciclovir or placebo 2 h after cell injection and daily thereafter in both procedures. At days 5 and 7 post-inoculation, mice from each treatment regimen were sacrificed and the gelatin sponge implants removed and evaluated for virus.

Our experiments showed a time-dependent increase in HCMV replication within the implanted gelatin sponge environment. During acute infection, virus titers increased from <30 to >1,000 pfu/mL over a nine-day period. Since peak virus replication appeared between 5 and 7 days after in vivo infection, we selected these times for drug evaluation end-points.

The immunofluorescent staining on gelatin sponge imprints and cross-sections showed nuclear and cytoplasmic inclusion antigens in all implants that had been inoculated with HCMV infected cells. Control specimens containing non-infected cells were negative for viral antigen. Strong fluorescence staining appeared in infected cells collected on day 4 post-inoculation. Both viral-specific immuno-staining

and viral growth studies showed that HCMV can survive and replicate in human cells within an *in vivo* gelatin sponge matrix. The presence of viable cells and increased virus titers in gelatin sponge samples indicated that nutrients were available to implanted cells via tissue fluids. Histology cross-sections also revealed that cells were entrapped inside pockets within the gelatin sponge.

In the acute infection model, virus replication in gelatin sponge implants from mice treated with ganciclovir was significantly lower than implants from saline treated animals. At day 5, three out of four mice in the placebo regimen displayed higher virus production than the drug treatment group. For day 7, virus titers from all four saline treated mice were again higher than three of the four mice receiving ganciclovir. Table 1 shows that mean virus titers from animals treated with saline were 4- to 5-fold higher than the drug treatment groups. The acute gelatin sponge infection model clearly demonstrated the antiviral effects of cytovene against HCMV replication.

Results from the cell-to-cell infection model also showed that gelatin sponge implants collected from the placebo group had significantly higher virus titers than animals which received drug treatments. Five days after cell injection, all six animals treated with ganciclovir demonstrated virus titers of <800 pfu/mL whereas four out of six animals which received saline treatments exhibited titers >4,000 pfu/mL.

The antiviral effect of ganciclovir is even more pronounced at day 7. All animals treated with drug displayed virus titers of <100 pfu/mL whereas saline treated animals presented titers of >3,000 pfu/mL. Table 2 shows that ganciclovir treated mice were 20- and 200-fold lower in mean virus titers compared to control mice on days 5 and 7, respectively. The antiviral effect observed in the cell-to-cell virus infection model was greater than the acute virus infection model (Tables 1 and 2) due to the former's higher virus titers that amplified the antiviral effect. In a later experiment when cells were acutely infected with a higher dose of virus, the observed drug effects were similar to the cell-to-cell infection model (Table 3). This experiment also showed that both ip and iv administered ganciclovir were equally effective in the mouse model.

Advantages and Other Applications of the Invention.

There are many advantages to using gelatin sponge mouse or rat models in viral diseases research. Unlike other matrices, gelatin sponge is approved by the FDA for in vivo use in humans and is readily available. The surgical procedure is not complicated to perform and foam pieces are easy to recover from mice. The procedure allows the use of low-cost outbred mice, or commonly available Sprague-Dawley rats, achieving significant savings when using the invention described herein. The mouse or rat model is well suited for the evaluation of *in vivo* efficacy of potential HCMV therapeutics and further experiments can be designed that may provide useful information for future clinical studies. But the system could be used with any mammal, including but not limited to the following commonly used research animals, rats, gineapigs, rabbits, dogs, monkeys, etc.

In addition to normal healthy research animals having a normal host defense, this system can also be used with any genetically immune-deficient mice such as nude mice, SCID or severe combined immunodeficiency mice. As well as in normal mice administered with various immuno-suppressive drug regimens such as cyclophosphamide, methotrexate, azathioprine, cyclosporine and anti-lymphocyte globulins. The procedures described herein can be modified for the study of other virus infections by using other cell types and viruses. In addition, the use of gelatin sponge as an in vivo matrix can be applicable to the study of drug metabolism and certain host immune mechanism.

The cells may be introduced into the gelatin sponge (inoculated) either before or after the gelatin sponge is introduced into a body cavity of a mammal. The cells may be infected with a virus (inoculated) either before or after the cells are introduced into the gelatin sponge. Obvious variations concerning the implanting of the gelatin sponge, introducing cells to the gelatin sponge, infecting the cells with the virus, before and after implanting the gelatin sponge are to be expected. The site of gelatin sponge implantation can also vary, it can be implanted at sites other than the subcutaneous route such as within the peritoneal cavity.

In addition to the cells described in the examples above, other target cells can be any cell of human origin; examples are human cell lines; primary cells obtained from human blood (such as lymphocytes and monocytes) as well as from human

tissues (such as hepatocytes from liver biopsies, macrophages from lung washing or peritoneal fluid).

Human cytomegalovirus is capable of replicating in human cells within gelatin sponge subcutaneously implanted in mice. The gelatin sponge infection mouse or rat model can provide valuable information on antiviral efficacy of lead anti-HCMV therapies.

Human cytomegalovirus is but one of many viruses that could be used in this system. Any other human virus that is difficult to culture in a non-human host, including but not limited to the following examples: human immunodeficiency virus (HIV), hepatitis C virus (HCV), and human papilloma virus (HPV). The types of antiviral drugs tested can also vary widely, antiviral agents can include organic or inorganic compounds with specific antiviral effect such as gancicovir. Also, products of natural or recombinant technology such as polyclonal and monoclonal antibodies, cytokines and interferon could be evaluated using this technology.

A more complete description of the model applied to Human immunodeficiency virus type 1 (HIV-1) follows.

HIV Model

H9 cells or peripheral blood monocuclear cells are infected with HIV-1 as previously described, see Chong *et al.,* "Bisheteroarylpiperazine Reverse Transcriptase inhibitor in Combination with 3'-Azido-3'-Deoxythymidine or 2', 3'-Dideoxycytidine Synergistically Inhibits Human Immunodeficiency Virus Type 1 Replication in Vitro," *Antimicrobial Agents and Chemotherapy*, Feb, 1994, p.288-293, and Pagano and Chong, " Synergistic inhibition of human immunodeficiency virus type 1 replication in vitro by two- and three-drug combinations of delavirdine, lamivudine and zidovudine" *Antiviral Chemistry & Chemotherapy* 8(4): 333-341 (1997), both incorporated herein by reference. Briefly, cells are exposed to HIV-1 3B or other clinical isolate such as HIV-1 JRCSF at multiplicity of infection of 0.01 for one and a half hours. Infected cells are then washed to remove free viral particles. Other HIV-1 susceptible cells derived from various lymphocyte cell lines such as CEM, MT-2, MT-4, A3.01, Hela CD4+ cell clones, Hela T4+, Molt- 4, and monocyte cell lines such as U-937, can also be used. In addition, both chronically infected lymphocyte (eg. ACH-2, CR10/N1T, H9/HTLV-IIIB) and monocyte derived cells (eg. U-1/HIV-1) can also be used. Infected cells are then injected into gelatin sponge pre-

implanted subcutaneously into outbred ICR mice. About 10^5 to 10^6 cells are injected per gelatin sponge implant. Implants are harvested 5 to 7 days later, homogenized in culture medium and supernatant samples are stored at -80°C. Samples are tittered for HIV-1 concentration by measuring p24 antigen concentration using an enzyme linked immunoassay (Coulter Diagnostic, Hialeah, Fla.). For drug evaluation, mice or rats are administered various dose levels of HIV-1 protease inhibitor such as ritonavir, PNU-140690 or HIV-1 reverse transcriptase inhibitor such as delarvidine or zidovudine. In other experiments various immune- deficient mice such as severe combined immune deficient (SCID), athymic (nu/nu) or triple deficient (bg\xid/nu; BIH-3) mice are used instead of the immune-competent ICR mice. Because of the potential health risk, all HIV-1 or HCV (described below) infected animals were housed under Biosafety Level 3 conditions.

A more complete description of the model applied to Hepatitis C virus (HCV), follows:

For HCV infection, either primary hepatocytes or cell lines may be used. Hepatocytes are harvested from liver samples by perfusion with collagenase. Isolated hepatocytes are cultured as adherent cells in culture wells pre-coated with collagen. For cell lines, sub-clone of MT-2 or other cells are cultured in suspension. These cells are infected with HCV by exposing to infectious serum obtained from donors shown to be positive for HCV infection. Alternatively, already infected hepatocytes or peripheral blood monocuclear cells are obtained from HCV positive donors. The HCV infected cells are injected into gelatin sponge previously implanted into outbred ICR mice. In some experiments, immune deficient mice such as SCID mice are used. Alternatively, Sprague-Dawley rats could be used, as could Sprague-Dawley rats treated with cyclophosphamide (see above under rat HCMV section). After 5 to 7 days, gelatin sponge samples are harvested. Samples are prepared for HCV detection and quantification by a real time quantitative polymerase chain reaction (PCR) assay. For evaluation of antiviral drug such as interferon-alpha, mice are administered various doses of interferon, twice a day, by intravenous route. After 5 to 7 days, gelatin sponge samples are harvested and stored at -80°C until assay.

Tables

Table 1. Effect of Ganciclovir (GCV) on Acute HCMV Infection in gelatin sponge Model.

Days After Treatment	Treatment Regimen*	Mean Virus Titer[1] pfu/mL (log_{10})	Log Reduction
5	Saline	735 (2.87)	0.72 (p = 0.06)[2]
	GCV	143 (2.15)	
7	Saline	859 (2.93)	0.63 (p = 0.03)
	GCV	199 (2.30)	

* Group of four ICR mice treated with GCV (ip 50 mg/kg/day).

[1] HEL cells were acutely infected with HCMV at MOI = 0.0007.

[2] Student's t-test: two-sample assuming unequal variances.

Table 2. Effect of Ganciclovir (GCV) on Cell-to-cell HCMV Infection in gelatin sponge Model.

Days After Treatment	Treatment Regimen*	Mean Virus Titer[1] pfu/mL (log_{10})	Log Reduction
5	Saline	5,028 (3.70)	1.34 (p = 0.03)[2]
	GCV	230 (2.36)	
7	Saline	6,510 (3.81)	2.33 (p = 0.005)
	GCV	30 (1.48)	

* Group of six ICR mice treated with GCV (ip 50 mg/kg/day).

[1] HCMV infected HEL cells were mixed with uninfected cells at 1:4 ratio.

[2] Student's t-test: two-sample assuming unequal variances.

Table 3. Effect of Route of Administration of Ganciclovir (GCV).

Days After Treatment	Treatment*	Route	Mean Virus Titer pfu/mL (log_{10})	Log Reduction
5	Saline	i.p.	4.19	
5	GCV	i.p.	2.73	1.46 (p<0.02)
5	GCV	i.v.	2.37	1.82 (p<0.02)
7	Saline	i.p.	3.95	
7	GCV	i.p.	2.57	1.38 (p<0.001)
7	GCV	i.v.	1.18	2.77 (p<0.001)

* Group of four or five ICR mice treated with GCV at 50 mg/kg/day.
HEL cells acutely infected with HCMV at MOI = 0.03.

CLAIMS

1. A process for selecting, measuring or evaluating compounds having antiviral activity comprising: exposing target or test compounds to virus growing in cells, where the cells are growing in gelatin sponge, where the gelatin sponge is implanted into a mammal, followed by selecting, measuring or evaluating the effect of the target compounds on the growth of the virus.

2. The process of claim 1 where the cells implanted into the gelatin sponge are selected from human cells.

3. The process of claim 2 where the human cells are selected from: primary cells obtained from human blood or cells from human tissues

4. The process of claim 3 where the cells from human blood are selected from lymphocytes and monocytes.

5. The process of claim 3 where the cells from human tissue are selected from human foreskin or hepatocytes from liver biopsies, macrophages from lung washing or peritoneal fluid.

6. The process of claim 1 where the virus is selected from human cytomegalovirus, human immunodeficiency virus (HIV), hepatitis c virus (HCV), or human papilloma virus (HPV).

7. The process of claim 6 where the virus is selected from human cytomegalovirus.

8. The process of claim 6 where the virus is selected from human immunodeficiency virus (HIV).

9. The process of claim 1 where the mammal is a rodent, a dog or a cat.

10. The process of claim 9 where the rodent is a mouse or a rat.

11. The process of claim 10 where the rodent is a mouse and the mouse is either a genetically immune-deficient mouse or a mouse treated with a immuno-suppressive drug regimen.

12. The process of claim 11 where the genetically immune-deficient mouse is selected from, nude mice, SCID or severe combined immunodeficiency mice, and mice administered with a immuno-suppressive drug regimens.

13. The process of claim 12 where the immuno-suppressive drug is selected from: cyclophosphamide, methotrexate, azathioprine, cyclosporine and a anti-lymphocyte globulin.

14. The process of claim 10 where the rodent is a rat and the rat is either a Sprague-Dawley rat or Sprague-Dawley rat treated with an immuno-suppressive drug regimens.

15. The process of claim 14 where the immuno-suppressive drug is cyclophosphamide.

16. The process of claim 3 where the cells from human tissues are derived from human embryonic lung tissue, where the virus is human cytomegalovirus, where the mammals are mice or rats.

17. The process of claim 4 where the cells are from lymphocyte cell lines, where the virus is HIV, where the mammals are mice or rats.

18. The process of claim 5 where the cells are selected from human foreskin fibroblast cells or hepatocytes harvested from liver tissue, where the virus is HCMV or HCV, where the mammals are mice or rats.

19. The process of claim 1 where the gelatin sponge is implanted into the mice or rats separately from when the virus is inoculated into the cells which are then inoculated into the gelatin sponge.

20. The process of using gelatin sponge for the evaluation of antiviral agents, comprising the following,

a) implanting gelatin sponge into a mammal,

b) infecting human cells with a virus,

c) injecting viral infected human cells into the gelatin sponge,

d) exposing the mammal to target compounds

e) evaluating the effect of target compounds on the viral infected cells in the gelatin sponge.

21. The process of claim 20 where the evaluation is comprised of selecting specific compounds having antiviral activity from the target compounds.

22. The process of claim 21 where the virus is selected from human cytomegalovirus (HCMV), human immunodeficiency virus (HIV) or hepatitus C virus HCV.

21. The process of claim 22 where the mammal is a rat or a mouse and the gelatin sponge is GELFOAM® and the GELFOAM® is implanted into the mouse subcutaneously.

INTERNATIONAL SEARCH REPORT

Ir ıtional Application No
PCT/US 98/17326

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 C12Q1/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US 5 785 964 A (NAUGHTON GAIL K ET AL) 28 July 1998 see claims 39-49 see column 2, line 60 - column 3, line 50 see column 8, line 50 - line 53 see column 28, line 59 - column 29, line 5 ---	1-21
X	US 5 601 975 A (BONYHADI MARK L ET AL) 11 February 1997 see claims see column 2, line 17 - line 28 see column 4, line 34 - line 42 --- -/--	1-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
16 November 1998	30/11/1998

Name and mailing address of the ISA	Authorized officer
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X	US 5 569 812 A (MONOSOV ANN ET AL) 29 October 1996 see claims see column 6, line 15 - line 34 see example IV	1-21
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X	EP 0 358 506 A (MARROW TECH INC) 14 March 1990 see claims 41-66 see page 3, line 29 - line 37 see page 6, line 48 see page 11, line 53 - line 61	1-21

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